FACTORS AFFECTING THE ACCURACY OF THE QUANTITATIVE DETERMINATION OF VITAMIN A

DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy,
in the Faculty of Pure Science,
Columbia University.

BY

MARY PENELOPE BURTIS

NEW YORK CITY
1928

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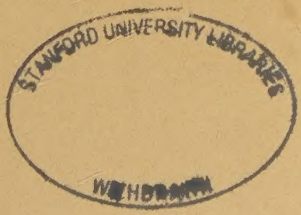

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FACTORS AFFECTING THE ACCURACY OF THE QUANTITATIVE DETERMINATION OF VITAMIN A

Introduction.

The quantitative determination of vitamin A by the rat growth method is now a highly important procedure. It is constantly being used to investigate both the chemical nature and properties of this factor, and its distribution in food materials. The general adoption of some standardized technique by all laboratories would result in the accumulation of more comparable data. This investigation was undertaken to study certain variable factors which affect the accuracy of the quantitative determination of vitamin A.

The term vitamin A^1 stands for a substance (or possibly a closely related group of substances) which is essential to growth and health. Concentrates, prepared from the unsaponfiable fractions of certain fats which consist chiefly of unsaturated monatomic alcohols of high molecular weight, possess the characteristic properties to a marked degree, but it has not yet been possible to decide whether vitamin A is a chemical individual identifiable with one of the sterols.

The most common criteria of its absence are cessation of growth on a diet adequate in all other respects and a more or less characteristic susceptibility to eye disease. But the latter is only one phase of a widespread weakening of the body tissues and increased susceptibility of several kinds. Its presence may be detected and its relative concentration measured quantitatively by means of sufficiently numerous and carefully controlled feeding experiments with rats amply provided with all other essential nutrients including vitamin D. When such provision is made, growth is believed to be a measure of the vitamin A present.

Development of the Modern Method. Drummond and Coward in 1920[2] and Zilva and Miura in 1921[3] in the development of the earliest quantitative technique, recognized the importance of observance of certain factors; namely, limiting the initial weight of the animal, ridding it of its surplus of vitamin A before using it to measure that factor in any material, feeding it a basal diet practically devoid of vitamin A, but containing all of the other dietary factors then known to be essential, and limiting its growth to some definite small gain.

Keeping these factors in view Sherman and Munsell in 1924[4] recommended the following method, "For the quantitative determination of the relative vitamin A content of foods, it is recommended that albino rats of known nutritional history be placed when 28 to 29 days of age upon a diet adequate in all other respects but free from vitamin A. After growth has ceased, the young rats are to be kept in individual cages and at least one of each litter should be continued on the basal vitamin A free diet until death, as a "negative control," while the others are to be fed graded portions of the food to be tested as their sole source of vitamin A, daily or at other suitable intervals during a test period of eight weeks." . . . "The unit recommended for numerical expression of results is that amount of vitamin A which when fed daily induces an average gain of 3 grams per week in a standard test animal under the conditions described." The essential features of this method were officially adopted by the United States Pharmacopoeia.[5] The specific regulations suggested as to the choice and control of experimental animals and procedure to be used will be discussed later in the light of more recent data accumulated in this laboratory. Other methods which have been suggested will not be considered in detail.[6, 7, 8, 9, 10, 11, 12]

Before giving the experimental procedure of this particular investigation, it is desirable to consider the bearing which the discovery of vitamin D had upon the quantitative methods for the determination of vitamin A. Steenbock and Nelson[13] found that under certain conditions, that is, with animals from

diets now recognized to have been low in vitamin D, their rats became stationary in weight on a diet deficient in vitamin A, but could be made to resume growth either when irradiated directly by a quartz mercury vapor lamp or when fed some irradiated food. The conclusion was that the first cessation of growth resulted from a deficiency of what is now designated as vitamin D. This inevitably raised the question as to how often in the past a shortage of this vitamin had limited the growth of animals which had been used for the quantitative determination of vitamin A.

While a final answer could not be given regarding their method when it was published in 1924,[4] Sherman and Munsell by a comparison of the weight records and autopsy findings of their experimental animals, concluded that "from these observations it would appear probable that the shortage of antirachitic substance in certain foods may become the factor limiting growth when considerable growth is sought or when the previous diet has been such as described by Steenbock [13]; but that when animals from previous diets such as ours are used and the gain in weight is limited to 3 grams per week, the weight curve is determined not by any antirachitic factor, but by vitamin A in the usual acceptance of the term. In any case, it now appears that the method can be so applied as to avoid the possibility of retardation of growth by shortage of the antirachitic factor since this may be supplied by irradiation either of the animal or of the food." In 1926 Hessler[17] concluded as a result of her experiments that under the conditions of the Sherman and Munsell method for the quantitative determination of vitamin A, additional vitamin D did not cause significantly greater growth. However, for the past few years much of the work done on vitamin A has been concerned with the pre-experimental store of vitamin D and the best method of supplementing this store when that has been believed to be necessary.

All of the variable factors which may affect the accuracy with which any given determination of vitamin A is made may be classified as those referable to either the animal itself,

its basal diet, the experimental procedure to which it is subjected, or the source of vitamin A. Of these, this investigation deals specifically with the best method of supplementing the pre-experimental store of vitamin D, the influence of the weight and sex of the animals, the most accurate rate of growth, and the best length of experimental period. The analysis of data with reference to these variable factors notes the effect of each upon the growth of the experimental animal.

It was evident that in a comprehensive study of certain of these variable factors the utilization of all of the data collected in this laboratory for several years would increase the value of the results. Including the work of several similarly trained experimenters would test the accuracy of the method in the hands of more than one individual, increase the number of cases, rule out the effect of season and year, represent many different sources of vitamin A, and would give some indication of the variability of results over a number of years in which the same original stock and breeding method were used. As it made possible a statistical treatment of the data, all which seemed sufficiently comparable were combined with those obtained by the writer in the discussion of results given later.[14, 15, 16, 17, 18, 19, 20, 21, 22, 23]

Experimental.

Comparison of Methods of Furnishing Vitamin D. Direct comparisons were planned to study the most efficient and satisfactory method of supplementing the animal's pre-experimental store of vitamin D. Albino rats, 28 to 29 days old, carefully matched as to litter, sex, and weight were placed on a diet practically free from fat soluble vitamins; casein 18%, dried brewer's yeast 10%, Osborne and Mendel salt mixture 4%, sodium chloride 1%, and cornstarch 67%; which is known as Diet 379a. Vitamin D was then furnished from 28 days of age until the end of the experimental period in one of the following ways:

1. By pre-experimental store.
2. By irradiating the animals 10 minutes daily at 22 inches with a mercury vapor quartz lamp.

3. By adding to the above diet 0.05% cholesterol which had been irradiated 30 minutes at 12 inches. This diet was freshly prepared each two weeks.
4. By irradiating the cornstarch of the above diet 379a, 100 grams at a time in a thin layer, 30 minutes at 12 inches. This diet was freshly prepared each two weeks.

As the pre-experimental store of vitamin D in the bodies of young is dependent upon the diet of the mothers, this comparison was made for animals reared on two diets which contained different amounts of vitamin D. The results were compared as to average gains made in the preliminary vitamin A depletion period, the average gains made in the eight weeks experimental period, during which time all of the animals were receiving 40 milligrams of dried spinach daily, six days each week, as a source of vitamin A, the appearance of the animals at the end of the depletion period and on autopsy, and the average weekly consumption of the basal diet. In the discussion of the results, these two comparisons as to previous diet are considered as Series I and Series II. The results are given in Table I.

(1) Series I. The discussion will treat first of the results obtained by placing animals from the diet containing one-third whole milk powder on the four experimental diets or procedures. When the gains in the depletion period were compared, sometimes a noticeable increase resulted from the furnishing additional vitamin D, doubtless because of the rapid gains made by some of the animals during this period, Sherman and Munsell having pointed out that differences may be expected in cases of rapid gain. In other cases, however, such differences were so slight that they could not be considered significant. Average gains made in the depletion period, as well as the time required to use up the surplus store of vitamin A were essentially the same in all four groups, Table I.

However, we are primarily interested in the effect on the growth in the eight weeks experimental period. When either the gains of litter mates or of all of the animals of this study were compared, it was clear that, considering the average

TABLE I.

Comparison of Methods of Furnishing Vitamin D.

Mother's Diet	Source of Vitamin D	Number of Animals	Average Weight at Beginning of Experimental Period. Grams	Average Gain in Depletion Period. Grams	Average Gain in Eight Weeks Experimental Period. Grams
13* Series I	Pre-experimental Store in Body	9 males 6 females	97 80	49 36	47 35
	Irradiation of the Animal	3 males 3 females	96 85	46 35	39 36
	Irradiated Cholesterol	4 males 6 females	86 87	41 40	49 34
	Irradiated Cornstarch	2 males 4 females	95 76	46 34	8 35
16† Series II	Pre-experimental Store in Body	4 males 5 females	64 61	24 23	55 42
	Irradiation of the Animal	4 males 4 females	82 73	46 34	31 37
	Irradiated Cholesterol	6 males 6 females	87 76	44 36	50 45
	Irradiated Cornstarch	6 males 3 females	79 74	39 37	61 38
Series I and Series II	Pre-experimental Store in Body	13 males 11 females	86 72	43 31	49 38
	Irradiation of the Animal	7 males 7 females	88 78	46 34	34 36
	Irradiated Cholesterol	10 males 12 females	86 81	42 38	49 39
	Irradiated Cornstarch	8 males 7 females	88 75	45 35	48 36

* Diet 13 consists of one-third whole milk powder, two-thirds ground whole wheat and two per cent of the weight of the wheat in sodium chloride.

† Diet 16 consists of one-sixth whole milk powder, five-sixths ground whole wheat and two per cent of the weight of the wheat in sodium chloride.

gains as well as the deviation of the individuals, there was no advantage in supplementing the vitamin D store in any particular way. Males receiving this vitamin from their pre-experimental store, direct irradiation, or irradiated cholesterol gained 40 to 50 grams in the eight weeks. The males receiving irradiated cornstarch, however, did not behave satisfactorily, gaining splendidly for five or six weeks and then losing rapidly and dying. The two which were able to complete the period showed at the end an average net gain of only 8 grams. When the gains of only females were compared, the results were identical in all four groups, that is, a gain of approximately 35 grams in the eight weeks with just as satisfactory behavior on irradiated cornstarch as with direct irradiation or irradiated cholesterol.

A tabulation of the condition of the eyes, hair, and flesh at the end of the depletion period did not show any marked superiority or inferiority of any one group. At this time (end of depletion period) opthalmia had appeared in from 50% to 65% of the animals and was very slight in most of these cases. The frequent criticisms in the literature as to the deleterious effect of direct irradiation of the animal did not seem warranted in the light of these comparisons. However, the frequent handling, draughts, and other annoyances attending irradiation must be reduced to a minimum and at best the method is so time-consuming that it might be well to discard it for this reason. The average weekly consumption of basal diet was found to be comparable in all groups. The rapid decline and death of the males on irradiated cornstarch was always attended by a decreased food consumption, but until the time of decline they ate as much as animals on the other diets.

(2) Series II. The results obtained with the animals from the diet containing one-sixth whole milk powder were compared in the same manner. All three groups receiving additional vitamin D gained on the average approximately 15 grams more during the depletion period than the group depending upon its pre-experimental store. While this excess is not great, it is consistent in all litters. The average time

required to deplete them of their store of vitamin A was several days less than that required for the animals on the better diet. The period required was no longer when additional vitamin D was furnished.

It is very important to note that in spite of the fact that these experimental animals do not seem to possess sufficiently large stores of vitamin D to support the full rate of growth in the early depletion period, yet all four groups made essentially the same gains in the eight weeks experimental period. The very large individual deviations, however, preclude any decision as to which of the four ways of furnishing vitamin D gave the better results. Rather, the differences can be said to be insignificant.

The hair, flesh, and eyes of all four groups showed no significant differences. Ophthalmia occurred at the end of the depletion period in approximately 65% of the animals. The rats were not as vigorous at this time as those from the diet containing the greater percentage of milk, and their hair and flesh were not as healthy. Previous extended studies comparing these two diets have shown that the one with the greater proportion of milk consistently produces more vigorous young. Upon autopsy only 25% of the 38 animals were free of infection. No group showed marked inferiority in this respect. It is important to note that this degree of infection is greater than that found in animals from the better diet, viz. 75% of the 37 animals from Diet 13 against 25% of the 38 animals from Diet 16 were found free from infection. The average weekly consumption of basal diet was essentially the same in all four groups.

(3) Series I and II Combined. When the results from Series I and Series II are combined in Table I, the average gains in the preliminary depletion period and in the eight weeks experimental period are essentially the same in all four groups, if the comparison is made within the same sex.

This evidence clearly shows that the pre-experimental store of vitamin D possessed by these young rats taken from mothers on diets containing either one-third or one-sixth

whole milk powder was usually sufficient to carry them through the quantitative determination of vitamin A in a successful manner. However, as the store of vitamin D is a variable factor which might in individual cases limit growth, it would seem wise to furnish an additional constant supply in some way. Judging from the experimental results of this investigation, the conclusions drawn by workers in other laboratories, and the relative convenience of the various ways in which this may be done, it would seem that a constant source of vitamin D can be furnished most easily, inexpensively, and effectively by the inclusion in the diet of some irradiated constituent which contains ergosterol, the substance now believed to be activated. Irradiated commercial cholesterol was found to be satisfactory in this investigation.

The Effect of Weight Upon Growth in the Experimental Period. Sherman and Munsell as a result of their investigations, found that when rats having the same previous dietary history were fed the vitamin A free food from the age of 28 to 29 days until death, the survival period was not necessarily influenced by the initial weight of the animal, within wide limits. A recent tabulation[24] of approximately 500 such "A-free controls" confirms this statement. While the tendency was for larger animals to survive longer, the differences within the limits described seem little more than a tendency. However, in order to avoid an error from this source, the authors thought it advisable to specify the use of rats whose initial weight at 28 days was not less than 35 nor more than 55 grams. Since animals carefully matched at 28 days may grow at very different rates in the preliminary depletion period, it is necessary to come to some conclusion as to how wide a variation in weight can be allowed at the beginning of the experimental period.

Numerous data were organized with reference to the weight at this time and the effect on growth in the experimental period noted. All available animals were grouped as small, medium-sized, and large. Weight in the medium-sized group was limited to from 75 to 100 grams at the begin-

ning of the experimental period. When the data permitted, each medium-sized animal was compared with a large and a small one which had been fed the same amount of vitamin A. However, as Table II indicates, such data were not always available and the comparison was more often made between individuals representing only two of the groups. Animals were always compared with one of the same sex. The results are shown as three separate comparisons each of strictly comparable groups of animals in Table II.

TABLE II.

Effect of Weight upon Growth in the Eight Weeks Experimental Period.

Number of Animals	Average Weight at Beginning of Experimental Period. Grams	Average Gain in Eight Weeks Grams	Probable Error	Differences and their Probable Errors	
When the rate of growth was 2-4 grams per week for eight weeks.					
81	65	26	±0.6		
81	87	20	±0.7	6 ±0.9	
28	89	25	±1.1		
28	115	15	±1.9	10 ±2.2	
13	69	30	±1.9		
13	89	22	±1.7	8 ±2.5	
13	113	14	±1.6	8 ±2.3	16 ±2.5
When the rate of growth exceeded 4 grams per week for eight weeks.					
105	62	47	±1.1		
105	85	45	±1.3	2 ±1.7	
24	89	50	±2.7		
24	113	29	±2.5	21 ±3.7	
9	64	48	±3.5		
9	90	42	±4.9	6 ±6.0	
9	109	24	±5.9	18 ±7.7	24 ±6.9

When the rate of growth was limited to 2 to 4 grams per week for eight weeks, 81 small animals grew significantly faster than 81 medium-sized animals which were identically

matched with the former group as to sex and amount of vitamin A. Twenty-eight medium-sized animals grew significantly faster than 28 large animals which were strictly comparable. Thirty-nine small, medium-sized, and large animals grouped into three comparable groups gave results in accord with the previous comparison.

Small and medium-sized animals growing at a rate exceeding 4 grams per week showed no significant difference. However, large animals grew at a significantly slower rate than medium or small animals.

A few data were available in which it was possible to compare gains made by animals whose initial weight at the beginning of the experimental period exceeded 100 grams in varying degrees. However, it was not possible to make this study for the "unit" rate of growth as all of the available animals grew at a rate exceeding 4 grams per week. Numbers were not sufficient to permit statistical treatment, but the differences were so great that there can be no doubt as to their significance. Sex was not allowed to exert an influence on results as the very few females used were compared with females. The results are shown in three separate comparisons, each of strictly comparable groups of animals in Table III.

TABLE III.

Effect of Extreme Weight upon Growth in the Eight Weeks Experimental Period.

Number of Animals	Average Weight at the Beginning of the Experimental Period, Grams	Average Gain in Eight Weeks Grams	Average Final Weight Grams
8	92	56	148
8	107	42	149
11	107	61	168
11	120	44	164
5	108	56	164
5	135	35	170

Animals of above 100 grams in weight which had been fed enough vitamin A to cause them to grow at a rate ex-

ceeding 4 grams per week for eight weeks, showed a tendency to gain inversely proportional to their weight.

Therefore, it is clear that the weight of the animal at the beginning of the experimental period determines to some extent the rate at which the animals will grow during the following eight weeks, and that the effect of weight upon growth increases more rapidly as the weight exceeds 100 grams. It is suggested that for the quantitative determination of vitamin A the size of the animal be restricted to from 75 to 100 grams at the beginning of the experimental period. There is probably less error involved in the use of animals smaller than this than there is in the use of those whose weights exceed 100 grams, unless of course, the small animal is a "runt" in a litter that is otherwise large in size.

Effect of Sex Upon Growth in the Experimental Period. Sherman and Munsell used males and females interchangeably since in their experiments started with four weeks old young, sex was found to have no appreciable influence upon the response of the animals to the vitamin A free food. A recent tabulation of approximately 500 animals placed on the vitamin A-free diet from 28 days until death confirms this. However, as numerous data involving growth of both sexes are now available, it was thought desirable to tabulate the results comparing the gains of males with the gains of females fed the same amounts of vitamin A. The results are recorded in Table IV.

Gains of males and females are compared at three rates of growth; the so-called "unit" rate of 2 to 4 grams per week for eight weeks, a rate of less than 2 grams per week, and a rate of more than 4 grams. Within each group each female always received the same amount of vitamin A as the male with which she was directly compared. The amount of vitamin A fed those growing at the greater rate of growth did not bear any constant ratio to the amount fed those gaining 2 to 4 grams per week for eight weeks. This limits the conclusions which can be drawn from these data. Within each group the data are organized as to the initial weight of the

animals, viz., first, when the initial weight of the male was less than that of the female, second, when it was within 10 grams greater, and third, when it was over 10 grams greater. This was done in order that any possible effect of weight on the results might be ruled out. These results are given in Table IV.

TABLE IV.

Effect of Sex upon Growth
in the Eight Weeks Experimental Period

Number of Animals	Average Weight at Beginning of Experimental Period. Grams	Average Gain in Eight Weeks Grams	Coefficient of Variation	Probable Error	Differences and their Probable Errors
Group I					
When the rate of growth was less than 2 grams per week for eight weeks					
12 males	83	5	222%	±1.9	
12 females	85	8	78%	±1.2	3 ±2.2
19 males	89	8	125%	±1.5	
19 females	84	8	133%	±1.6	
19 males	100	9	119%	±1.6	
19 females	83	11	80%	±1.3	2 ±2.2
Group II.					
When the rate of growth was 2-4 grams per week for eight weeks.					
26 males	76	26	38%	±1.3	
26 females	84	20	39%	±1.0	6 ±1.8
92 males	83	24	32%	±0.6	
92 females	79	22	35%	±0.6	2 ±0.85
30 males	92	25	34%	±1.0	
30 females	71	25	34%	±1.0	
Group III.					
When the rate of growth exceeded 4 grams per week for eight weeks.					
41 males	68	61	45%	±2.9	
41 females	76	45	42%	±2.0	16 ±3.5
117 males	77	57	40%	±1.4	
117 females	73	42	35%	±0.9	15 ±1.7
67 males	93	58	33%	±1.6	
67 females	68	43	35%	±1.2	15 ±2.0

Animals were then selected from these data by imposing the following restrictions: first, initial weight was limited to from 70 to 110 grams, and usually to from 75 to 100 grams at the beginning of the experimental period, second, those growing at the greater rate of growth were divided into two groups. Dutcher[11] has suggested a rate of 6 grams per week for five weeks which will be discussed later. In this connection, it was desired to see if the gains of males and females were comparable at that rate, hence animals growing at a more nearly normal rate were excluded from the more restricted comparison. As the Dutcher standard suggests a five weeks experimental period, data are given for both a five and an eight weeks experimental period for the same rats, in Table V.

TABLE V.

Effect of Sex upon Growth
in the Five and Eight Weeks Experimental Period.

No. of Animals	Average Weight at Beginning of Experimental Period. Gms.	Average Gain in Five Wks. Grams	P. E.	Differences and their P. E.	C. V.	Average Gain in Eight Wks. Grams	P. E.	Differences and their P. E.	C. V.
I. Selected from Group II, Table IV, Initial weight 75 to 100 grams.									
36 males	90	18	±0.8		40%	24	±0.8		30%
36 females	86	16	±0.8	2 ±1.1	43%	22	±0.9	2 ±1.1	35%
II. Selected from Group III, Initial weight 70 to 110 grams.									
Rate of growth approximately 6 grams per week for the first five weeks.									
36 males	89	37	±1.1		28%	49	±1.6		30%
36 females	85	27	±0.9	10 ±1.4	30%	36	±1.2	13 ±2.0	29%
Rate of growth approaching normal.									
11 males	83	71	±2.9		20%	96	±3.7		19%
11 females	79	48	±2.7	23 ±4.0	28%	63	±3.1	33 ±6.6	24%

When the rate of growth was less than 2 grams per week no significant difference was detected between the gains of males and females. This may have been partially due to the limited number of animals available. However, when the rate of growth was 2 to 4 grams per week, and the number of animals used was greater it was shown that when the males

were smaller than the females they showed a tendency to gain faster than the females. As the size of the male exceeded that of the females, the difference grew less significant and finally disappeared when the initial weight of the male exceeded that of the female by 20 grams. Still larger numbers of animals growing at a rate of growth exceeding 4 grams per week showed that beyond a doubt the males grew significantly faster than the females on identical amounts of vitamin A when the amount of the vitamin fed is distinctly larger than in our usual experiments for the quantitative determination of vitamin A. Variability in the growth of males is no greater than in the growth of females as judged by the coefficient of variation, if the animals are growing at 2 grams per week or more for eight weeks.

The more carefully selected animals in Table V gave the following results. As had been found in Table IV, males and females growing at 2 to 4 grams per week for eight weeks, grew at the same rate. Males grew significantly faster than females when the rate of growth was approximately 6 grams per week for the first five weeks, whether the results were considered at the end of five or at the end of eight weeks. The actual difference in grams was less at five weeks as would be expected. As growth approaches normal, the gains of males exceeded the gains of females even more.

Inasmuch as the weight of the male usually exceeds that of the female at the beginning of the experimental period, we may conclude that at the proposed "unit" rate of growth of about 3 grams per week, males and females may be used interchangeably. The use of standards suggesting greater rates of growth will introduce errors unless the sexes are carefully matched.

Rate of Growth. Following the earlier suggestions that some definite rate of growth be used for comparison, Sherman and Munsell studied the behavior of comparable animals at various levels of growth and as a result suggested a rate of approximately 3 grams per week, as has been discussed previously. Greater rates of growth were found to be less sen-

sitive, smaller differences resulting from the addition of equal amounts of vitamin A as normal growth was approached. A rate between the Sherman and Munsell and Normal Growth standards has been suggested by Dutcher and co-workers[11]. They state that, "We have the feeling that normal growth standards are not capable of detecting small differences in the vitamin constituent of foods while we are also somewhat disturbed when we apply the Sherman-Munsell standard, for the reason that we are in doubt regarding the influence of lowered resistance and infection on the results. While we are not yet ready to advocate it, we are inclined to favor the 'half normal' standard of Javillier." This standard, according to Dutcher, suggests a rate of growth of 6 grams per week for five weeks. Therefore, to compare its variability with that of the Sherman-Munsell standard, it is necessary to study matched animals growing at the two rates of growth, at the end of five as well as at the end of eight weeks. The results are given in Table VI. Since it has been shown,

TABLE VI.

Variability in Gains at Two Rates of Growth.

Fed enough vitamin A food to gain 2-4 grams per week for eight weeks.				Fed twice as much vitamin A food.			
Number and Sex	Average Weight at Beginning of Experimental Period. Grams	Average Gain Grams	C. V.	Number and Sex	Average Weight at Beginning of Experimental Period. Grams	Average Gain Grams	C. V.
Compared at the end of eight weeks.							
52 females	73	25	25%	52 females	71	38	24%
23 males	81	26	47%	23 males	81	47	28%
18 males	72	26	28%	18 females	69	36	28%
11 females	82	27	31%	11 males	88	55	31%
104	76	26	26%	104	74	42	30%
Compared at the end of five weeks							
52 females	73	19	46%	52 females	71	27	31%
23 males	81	16	54%	23 males	81	33	31%
18 males	72	17	42%	18 females	69	25	26%
11 females	82	19	38%	11 males	88	40	33%
104	76	18	47%	104	74	29	34%

Tables IV and V, that males grow significantly faster than females fed the same amount of vitamin A, if the rate of growth exceeds 4 grams per week, the comparisons were first made in such a manner that sex could not influence the results and then averaged together. Weights were evenly matched at the beginning of the experimental period.

When the coefficient of variation of the average gain of 52 females fed enough vitamin A food to gain 2 to 4 grams per week for eight weeks was compared with that of the gain of 52 matched females fed twice as much vitamin A, the coefficients of variation were of the same magnitude, that is, approximately 25%. Smaller groups in which males grew at one rate and females at the other, gave no greater variability in one group than another. When the animals were all considered together, the coefficients of variation of the two groups were of the same order of magnitude.

When the same animals were compared at the end of five weeks, which is the length of experimental period suggested by Dutcher, the coefficient of variation was always less at the greater rate of growth. Its magnitude remained about the same for the Dutcher standard, but increased for the Sherman-Munsell standard. Therefore, if the Sherman-Munsell method is applied in full, that is, as regards the length of experimental period, as well as rate of growth, the variability of results is no greater than when the Dutcher standard is applied. A fuller discussion of length of experimental period is given later.

In addition to the factor of variability in growth at the two rates, it is necessary to consider how much the rate of growth is increased by feeding twice as much vitamin A. When matched females grew at the two rates, doubling the amount of vitamin A increased the gain only 1.5 times. When males were matched with females, the increase in gain was 1.4 times, which is essentially the same as in the preceding case. This is what is to be expected, since the data on the influence of sex proved that males and females can be used interchangeably at the lower rate of growth. When males grew at the

lower rate and were matched with males fed twice as much vitamin A, the increase in gain was 1.8. When females were fed the smaller amount and males twice as much, the increase in gain was doubled. The total 104 animals grew 1.6 times as fast as the 104 which had received one-half as much vitamin A. Essentially the same results are given at the end of five weeks. Therefore, judging from this standpoint, it would seem that the Dutcher standard would not give quite as accurate an analysis of the vitamin content of the food, as does the Sherman-Munsell method. As this comparison of standards involves length of experimental period, conclusions will be waived until that factor has been discussed.

Length of Experimental Period. The interpretation of results from any one laboratory, or from different sources must have as a comparable basis some definite length period during which growth has been measured. As some laboratories have adopted a five weeks experimental period, it was thought to be of value to compare results obtained when data for identical animals were considered at the end of a five as well as an eight weeks period. The variability of gains, and the rate of gain per week were compared for the two length periods at the two different rates of growth which we have discussed. The animals used in the comparison of a five and an eight weeks experimental period are those which have already been given in Tables V and VI. In Table VII they are re-organized to bring out the factor of length of experimental period.

When the rate of growth was 2 to 4 grams per week for eight weeks, the coefficient of variation was always increased around 10% or more when the length of experimental period was decreased from eight to five weeks. The rate of growth per week increased approximately 0.4 gram. Therefore experiments using this rate of growth and terminated at the end of five weeks indicate a slightly higher vitamin A content of the food tested than if continued until the end of eight weeks.

TABLE VII.

Comparison of a Five and an Eight Weeks Experimental Period.

Number and Sex	Average Weight at Beginning of Experimental Period. Grams	Average Gain in Five Weeks Grams	Coefficient of Variation	Average Gain in Eight Weeks Grams	Coefficient of Variation
When the rate of growth was 2 to 4 grams per week for eight weeks.					
36 males	90	18	40%	24	30%
36 females	86	16	43%	22	35%
72	88	17	42%	23	32%
When the rate of growth was approximately 6 grams per week for the first five weeks					
36 males	89	37	28%	49	30%
36 females	85	27	30%	36	29%
72	87	32	34%	42	35%
When the rate of growth approached normal.					
11 males	83	71	20%	96	19%
11 females	79	48	28%	63	24%
22	81	59	31%	79	30%
Fed enough vitamin A to gain 2 to 4 grams per week for eight weeks.					
52 females	73	19	46%	25	25%
23 males	81	16	54%	26	47%
18 males	72	17	42%	26	28%
11 females	82	19	38%	27	31%
104	76	18	47%	26	26%
Fed twice as much vitamin A food.					
52 females	71	27	31%	38	24%
23 males	81	33	31%	47	28%
18 females	69	25	26%	36	28%
11 males	88	40	33%	55	31%
104	74	29	34%	42	30%

If approximately twice as much growth was required of the animals, reducing the period to five weeks did not cause such an increase in the coefficient of variation. Furthermore,

if this rate of growth is to be used, the difference in the gain between males and females is not so great at this time as at the end of eight weeks. It follows that the rate of growth per week is increased more for the males than for the females. The increase for the average of the two exceeded the increase in gain per week found when the Sherman-Munsell rate of growth is terminated at the end of five weeks. As growth more nearly approaches normal, the gain per week is correspondingly greater for the first five than for the first eight weeks.

All factors considered, the Sherman and Munsell method gave the most accurate results in an eight weeks period. The Dutcher method gave just as small coefficients of variation in a five as an eight weeks period. However, at the latter rate of growth, males and females cannot be used interchangeably, and as small concentrations of the vitamin cannot be detected.

Summary

In view of the present degree of standardization of the modern developed rat growth method for the quantitative determination of vitamin A, a study of the factors affecting its accuracy was considered to be of value. The variable factors considered were: different ways of supplying the animal with additional amounts of vitamin D, the influence of the weight and sex of the animals upon their growth in the experimental period, the most accurate rate of growth, and the best length of experimental period.

As a result, it was possible to make the following positive contributions to our knowledge concerning the quantitative determination of vitamin A:

It was found that animals from mothers on a diet containing either one-third or one-sixth whole milk powder usually had stores of vitamin D sufficient to carry them through the recommended procedure in a successful manner. However, as the store was found to vary considerably, it was suggested that in the future additional vitamin D be furnished. Judging by direct comparisons in which this was done by irradiat-

ing either the animal or the starch of the basal diet, or by feeding irradiated commercial cholesterol, it was decided that the latter was the more satisfactory method. It cannot be said that either of the other two methods was consistently detrimental.

The weight of the animal at the beginning of the experimental period was found to be a factor in its growth during the eight weeks following. Small animals, that is, those under 75 grams, usually grew at a greater rate than medium-sized animals which weighed between 75 and 100 grams. Medium-sized animals grew faster than large animals which weighed over 100 grams. The difference in the rate of growth of small and large animals was statistically significant. This effect of weight became more marked as the initial weight of the animal, i. e., its weight at the beginning of the experimental period, exceeded 100 grams. The growth of animals of this weight was found roughly to be inversely proportional to the initial weight. It is suggested that, for the quantitative determination of vitamin A, the weight of the animal at the beginning of the experimental period be limited to from 75 to 100 grams.

Males and females grew at essentially the same rate, if their growth was limited to less than four grams per week, whether the results were considered at the end of five or eight weeks. At a rate of growth exceeding four grams per week, the males gained significantly faster than the females on the same amount of vitamin A. This difference was not eliminated by reducing the experimental period to five weeks. The coefficient of variation of the gains of males was of essentially the same magnitude as the coefficient of variation of females if the rate of gain exceeded 2 grams per week.

When the variability of the gains at the end of the eight weeks experimental period was judged by the coefficient of variation, the variations were of the same magnitude whether animals grew at approximately 3 grams per week for eight weeks (as suggested by Sherman and Munsell), or at approximately 6 grams per week for five weeks (as suggested by

Dutcher and Honeywell). Compared at the end of five weeks, the coefficient of variation increased for the gains made by animals growing at the slower rate and remained essentially the same for those growing faster. The rate of growth per week for the first five weeks slightly exceeded the rate per week for eight weeks, when animals grew at 2 to 4 grams per week, and more markedly exceeded it when the rate was approximately 6 grams per week for the first five weeks. The shorter test period, therefore, tends to give the material tested a higher numerical rating in vitamin A value.

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